REMARKS

The Claims

Claims 26-44 have been cancelled without prejudice or disclaimer and new claims 45-70 have been added.

New Claim 45 recites a method of treating cancer comprising administering to a patient in need thereof an anti-neoplastic therapeutic agent conjugated to a monoclonal antibody or fragment thereof which binds a human stem cell factor receptor and inhibits binding of human stem cell factor to the receptor. Support for Claim 45 is found in the specification at p. 17, line 25 to p. 18, line 9 and p. 19, lines 3-16.

New Claims 46-58 correspond to claims which have been previously presented.

New Claims 59-62 are directed to antibody compositions and find support in the specification at p. 22, lines 2-13.

New Claims 63-68 are directed to anti-neoplastic therapeutic agents selected from one or more of a radioisotope, a toxin, an antitumor drug, an antibiotic, and a cytostatic drug. These claims and find support in the specification at p. 18, lines 9-14 and p. 38, line 6 to p. 39, line 25.

New Claims 69 and 70 are directed to a cancer which is a solid tumor or leukemia. Support for these claims is found at p. 38, lines 3-7.

Rejections under 35 U.S.C. 112

Claims 26-44 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite.

Claim 26 is alleged to be indefinite because it is unclear whether the receptor-containing cells are cancerous. It is believed that the rejection has been overcome as Claim 26 has been cancelled and new Claim 45 recites a method of treating cancer in a patient "in need thereof".

Claim 32 is alleged to be indefinite for recitation of the term "essentially indefinitely". The rejection is moot as Claim 32 has been cancelled.

Claim 44 is alleged to be indefinite as it is unclear what substances are encompassed by the term "Tween". It is believed that the rejection is overcome as Claim 44 has been cancelled and new Claim 62 recites a "detergent".

Claims 26-44 are rejected under 35 U.S.C. 112, first paragraph, for failing to comply with the written description requirement. The Examiner alleges that there is not adequate written description in the specification for separate chemotherapeutic agents and the antibody. Without acquiescing to the rejection and solely to advance prosecution, Applicants have cancelled Claims 26-44 thereby rendering the rejection moot.

Claims 26-44 are rejected under 35 U.S.C. 112, first paragraph, for failing to comply with the written description requirement. The Examiner alleges that there is not adequate written description in the specification for "a receptor recognized by human stem cell factor". Without acquiescing to the rejection and solely to advance prosecution, Applicants have cancelled Claims 26-44 thereby rendering the rejection moot.

Claim 27 is rejected under 35 U.S.C. 112, first paragraph, for failing to comply with the written description requirement. The Examiner alleges that there is not adequate written description in the specification for "an epitope" recognized by the antibody produced by the hybridoma cell line HB 10716. It is argued that the specification does not disclose "what linear residues or region or what combinations of residues would make up the amino acids in the epitope for the antibody to bind to."

Without acquiescing to the rejection and solely to advance prosecution, Applicants have cancelled Claim 27 thereby rendering the rejection moot. New Claim 45 is directed to a method of treating cancer by administering an anti-neoplastic therapeutic agent conjugated to a monoclonal antibody or fragment thereof wherein the monoclonal antibody or fragment binds to a human stem cell factor receptor and inhibits stem cell factor binding to the receptor.

Claims 26-44 are rejected under 35 U.S.C. 112, first paragraph, for failing to enable any person skilled in the art to make and use the invention commensurate in scope with the claims. The Examiner alleges that specification does not reasonably provide enablement for a method of treating any cancer or any cell that has any receptor recognized by stem cell factor and argues that carrying out the invention as claimed would require undue experimentation (citing *Ex parte Forman* 230 USPQ 546 (BPAI 1986)).

Without acquiescing to the rejection and solely to advance prosecution, Applicants have cancelled Claims 26-44. New Claim 45 is directed to a method of treating cancer by administering an anti-neoplastic therapeutic agent conjugated to a monoclonal antibody or fragment thereof wherein the monoclonal antibody or fragment thereof binds to a human stem cell factor receptor and inhibits stem cell factor binding to the receptor. It would not require undue experimentation to identify those antibodies which could be administered to a patient. For example, one skilled in the art could readily carry out a competition binding experiment such as that described in Example 4 to determine whether a given antibody inhibits binding of stem cell factor to a human stem cell factor receptor. With these antibodies in hand, one skilled in the art could readily introduce an anti-neoplastic therapeutic agent by conjugation using any number of publicly available methods.

The Examiner cites publications by Curti (Crit. Review in Oncology/Hematology 14, 29-39 (1993)) and Jain (Scientific American 271, 58-65 (1994)) to support the position that treating tumors using chemotherapeutic agents and antibodies has met with limited success and presents significant technical hurdles. While the development of a treatment for a disease can certainly meet with hurdles and setbacks, there is nothing in the publications cited by the Examiner to suggest that treating cancer with the claimed antibodies would entail undue experimentation. The specification teaches various antibodies and anti-neoplastic therapeutic agents one may use and discloses that certain cancers, such as solid tumors and leukemia, are amenable to treatment using the claimed antibodies. For example, the presence of human stem cell factor receptors on certain tumor cell lines, such as OCIM1, a human erythroleukemia cell lines, teaches one skilled in the art that leukemia could be treated using the claimed antibody conjuates.

Rejection under 35 U.S.C. 103

Claims 26 and 29-44 are rejected under 35 U.S.C. 103(a) as being obvious over Gadd et al. (Leukemia Res. 9, 1329-1336 (1985)) as evidenced by Broudy et al. (Blood 79, 338-346 (1992)) and further in view of Cambareri et al. (Leukemia Res. 12, 929-939 (1988)), Hara et al. (Proc. Natl. Acad. Sci. 84, 3390-3394 (1987)), Riechmann et al. (Nature 332, 323-327 (1988)) and Byars et al. (Vaccine 5, 223-228 (1987)). The Examiner argues it would have been obvious to treat leukemia patients with a conjugate of an antibody that inhibits binding of stem cell factor to its receptor and decreases the growth rate of the cells. The Gadd et al. and Cambareri et al. publications were cited as disclosing the antibody YB5.B8 which was subsequently shown by Broudy et al. to bind human c-kit and inhibits stem cell factor binding to

c-kit. Hara et al. was cited as teaching complete suppression of human leukemia cells by immunotoxins. Riechmann et al. was cited as disclosing methods for making humanized antibodies and Byars et al. for disclosing use of Tween 80 in protein formulations.

Without acquiescing to the rejection and solely to advance prosecution, Applicants have cancelled Claims 26 and 29-44. New Claim 45 is directed to a method of treating cancer by administering an anti-neoplastic therapeutic agent conjugated to a monoclonal antibody or fragment thereof wherein the monoclonal antibody or fragment thereof binds to a human stem cell factor receptor and inhibits human stem cell factor binding to its receptor. The claimed antibodies are different from YB5.B8 in part because they inhibit binding of human stem cell factor to the receptor and inhibit cell growth of receptor-containing cells. Ashman et al. (J. Cell Physiol 158, 545-554 (1994) attached hereto as Exhibit A) states that SR-1, the antibody produced by the hybridoma cell line ATCC No. HB 10716, blocked binding of SLF (*steel* factor, an alternative name for stem cell factor) to its receptor whereas YB5.B8 had minimal effects on SLF binding (abstract, p. 545). With respect to the Examiner's assertion that Broudy et al. state that YB5.B8 inhibited stem cell factor binding to c-kit, it should be noted that YB5.B8 inhibited binding of murine, but not human, stem cell factor to human stem cell factor receptor (see Ashman et al. at p. 551, right hand column). In addition, SR-1 inhibited growth of receptor-containing cells whereas YB5.B8 did not (abstract p. 545). The claimed invention would not have been obvious since the YB5.B8 antibody does inhibit either human stem cell factor binding to its receptor or growth of receptor-containing cells. Accordingly, there was no reasonable expectation that such an antibody would be effective in cancer therapy. It is believed that the rejection may be withdrawn.

CONCLUSION

Claims 45-70 are in condition for allowance and an early notice thereof is solicited.

The Commissioner is hereby authorized to charge any additional fees or credit any overpayments to Deposit Account No. 01-0519.

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JOURNAL OF CELLULAR PHYSIOLOGY 158:545–554 (1994)

Epitope Mapping and Functional Studies With Three Monoclonal Antibodies to the *C-KIT* Receptor Tyrosine Kinase, YB5.B8, 17F11, and SR-1

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Three monoclonal antibodies (MAbs) to the human c-*kit* receptor tyrosine kinase ($P145^{c\text{-}kit}$), derived in independent laboratories, have been extensively used in studies of c-*kit* expression and the role of its ligand, *steel* factor (SLF), in hemopoiesis and mast cell differentiation and function. In this study, the relationship between the epitopes they identify, and their effects on SLF binding, receptor internalization, and signal transduction are compared.

Epitope mapping studies carried out on the high $P145^{c\text{-}kit}$-expressing cell line HEL-DR showed that SR-1 identifies an epitope independent of those bound by YB5.B8 and 17F11, while the latter two antibodies bound to distinct but interacting epitopes. SR-1 potently blocked the binding of SLF to $P145^{c\text{-}kit}$ on these cells and also on cells of the factor-dependent line MO7e. In contrast, YB5.B8 and 17F11 had minimal effects on ligand binding. Conversely, SLF partially blocked the binding of SR-1 and YB5.B8 to cells, while binding of 17F11 was actually enhanced by SLF on some target cells. Preincubation of HEL-DR and MO7e cells with MAbs prior to exposure to SLF revealed that 17F11 itself brought about partial down-regulation of $P145^{c\text{-}kit}$ and did not inhibit SLF-mediated down-regulation. SR-1 caused minimal down-regulation and inhibited SLF-mediated receptor internalization. YB5.B8 had minimal effects on either cell line in this assay. To determine whether the antibodies had any agonist activity, they were compared with SLF for their ability to bring about receptor phosphorylation in intact MO7e cells. All three antibodies induced detectable tyrosine phosphorylation with 17F11 being the most effective, while YB5.B8 was the least effective. Finally, the ability of the antibodies to influence the proliferation of the MO7e cells was examined. As expected, SR-1 potently inhibited the proliferative response to SLF, while 17F11 weakly inhibited and YB5.B8 had negligible effect. In the absence of SLF both 17F11 and YB5.B8 displayed very weak but reproducible agonist activity.

The *c-kit* proto-oncogene product, $P145^{c\text{-}kit}$, is a 145–150 KDa cell surface glycoprotein with intrinsic tyrosine kinase activity (Yarden et al., 1987) and serves as the receptor for the cytokine variously known as stem cell factor (SCF), mast cell growth factor (MGF), kit ligand (KL) and *steel* factor (SLF) (Flanagan and Leder, 1990; Huang et al., 1990; Anderson et al., 1990; Zsebo et al., 1990). The mapping of *c-kit* to the *W* locus in the mouse (Chabot et al., 1988; Geissler et al., 1988) and of SLF to the *Steel* locus (Flanagan and Leder, 1990; Huang et al., 1990; Zsebo et al., 1990; Copeland et al., 1990) demonstrated the importance of this receptor-ligand system in the processes of hemopoiesis, mast cell differentiation, melanogenesis, and germ cell development. Several monoclonal antibodies (MAbs) recognizing the extracellular domain of $P145^{c\text{-}kit}$ have been described (Lerner et al., 1991; Bühring et al., 1991a, 1993; Broudy et al., 1992a; Blechman et al., 1993; Reisbach et al., 1993) and have proved valuable in analyzing the cellular distribution of the receptor, isolation of $P145^{c\text{-}kit}$-positive cells, and the study of $P145^{c\text{-}kit}$ function.

MAb YB5.B8 (IgG1) was raised against blast cells from a patient with acute myeloid leukaemia (AML) of

Received August 17, 1993; accepted October 25, 1993.

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M1 type and selected for further study based on its failure to bind to autologous Epstein-Barr virus-transformed B cells or to normal peripheral blood cells (Gadd and Ashman, 1985). The antibody was subsequently shown to identify a 145–150 KDa protein which was also present on tissue mast cells (Ashman et al., 1987; Mayrhofer et al., 1987) and a small proportion of normal bone marrow mononuclear cells including hemopoietic progenitor cells that gave rise to colonies in semisolid media, CFU-C (Cambareri et al., 1988). The mapping of the *c-kit* gene to the murine *W* locus (Chabot et al., 1988; Geissler et al., 1988), known to be of importance in hemopoiesis (Russell, 1979) and to be strongly expressed in mast cells (Nocka et al., 1989), suggested that the antigen identified by YB5.B8 might be $P145^{c\text{-}kit}$. This was confirmed in sequential precipitation and other experiments (Lerner et al., 1991).

MAb 17F11 (IgM) was also raised against blast cells from a patient with AML and selected for its lack of reactivity with normal peripheral blood cells as well as its binding to a minor population of bone marrow mononuclear cells (Bühring et al., 1991a). This pattern of binding, which was similar to the previously reported pattern of expression of *c-kit* mRNA in AML and normal marrow (Wang et al., 1989), suggested that the target antigen might be $P145^{c\text{-}kit}$. Demonstration that 17F11 bound to murine 3T3 cells transfected with human *c-kit* cDNA proved this to be the case (Bühring et al., 1991a).

MAb SR-1 (IgG2a) was produced by immunization of mice with cells of the OCIM1 line. This antibody was directly identified as an anti-$P145^{c\text{-}kit}$ MAb by virtue of its ability to block the binding of ^{125}I-labeled SCF to OCIM1 cells (Broudy et al., 1992a).

These MAbs have been extensively used to study $P145^{c\text{-}kit}$ expression and function. All three MAbs have demonstrated the presence of $P145^{c\text{-}kit}$ on a minority of bone marrow cells, including most cells of the progenitor compartment (Cambareri et al., 1988; Ashman et al., 1991; Bühring et al., 1991a; Papayannopoulou et al., 1991; Broudy et al., 1992a). More recently, SR-1 and YB5.B8 have been used to isolate human bone marrow hemopoietic "stem" cells capable of initiating long-term in vitro hemopoiesis in cytokine supplemented cultures (Briddell et al., 1992; Simmons et al., 1994) and stroma-dependent Dexter cultures (Simmons et al., 1994). MAb YB5.B8 has been extensively used in studies of mast cell development and function (e.g., Valent et al., 1989, 1992; Irani et al., 1992; Bradding et al., 1992; Nilsson et al., 1993). SR-1 has also been used to study mast cell and basophil function (Columbo et al., 1992).

The reported extent and pattern of binding of the MAbs to leukemic cells vary. In early experiments, approximately 25–30% of AML specimens were scored as positive for YB5.B8, with no binding to acute lymphoblastic leukemia (ALL), chronic granulocytic leukemia (CGL), or chronic lymphocytic leukemia (CLL) cells detected (Gadd and Ashman, 1985; Ashman et al., 1988). These values underestimate the true frequency of YB5.B8 positivity due to the relatively low sensitivity of the immunofluorescence used at the time (L.K. Ashman, unpublished data). MAb 17F11 was found to bind to blast cells from most patients with AML and CGL in blast crisis, but not to ALL specimens (Bühring et al., 1991a).SR-1 was found to be positive on some ALL cell lines and on blast cells of all 20 primary AML specimens examined (Broudy et al., 1992a,b). Whether these data reflect true differences in specificity of the antibodies, or, as seems more likely, differences in the sensitivity of the detection methods employed, remains to be determined.

It has been shown that MAbs SR-1 and YB5.B8 identify distinct epitopes on the $P145^{c\text{-}kit}$ molecule (Broudy et al., 1992a) but, other than that, no direct comparisons of these three MAbs have been reported. We here describe the results of collaborative studies in which we have directly compared these antibodies in epitope mapping and functional assays.

MATERIALS AND METHODS

MAbs

In preliminary studies all three anti-$P145^{c\text{-}kit}$ MAbs were used as dilutions of ascites after titration to determine their relative potency. YB5.B8 ascites was saturating down to a 1/1,000 dilution in immunofluorescence assays on HEL-DR cells, compared with 1/200 for 17F11 and >1/5,000 for SR-1. For the experiments shown in this paper, except where stated otherwise, purified antibodies were used throughout at 2–10 μg/ml, as indicated. MAb YB5.B8 (IgG1) was purified from culture supernatant by affinity chromatography on Protein A-Sepharose, and SR-1 (IgG2a) was purified from ascites using Protein G-Sepharose. MAb 17F11 was purified on a Hi Load 16/60 Superdex 200 prep-grade column as described previously (Bühring et al., 1991a). Negative control antibodies were SAL-2 (IgG1), SAL-3 (IgM), and SAL-5 (IgG2a) (O'Connor and Ashman, 1982). MAb 3D6 (anti-$P145^{c\text{-}kit}$) was obtained from Boehringer-Mannheim. The antiphosphotyrosine MAb 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cells, cell lines, and cytokines

Normal human bone marrow was obtained with informed consent, and with the approval of the Royal Adelaide Hospital Ethics Committee, from adult volunteers. The mononuclear fraction was prepared by Ficoll-Paque density gradient centrifugation. HEL-DR, a subline of the erythroleukemia line HEL (Martin and Papayannopoulou, 1982), selected for its expression of HLA class II antigens, was kindly provided by Dr. Beverly Torok-Storb, Fred Hutchinson Cancer Research Center, Seattle. This line was found to express high levels of $P145^{c\text{-}kit}$ (L.K. Ashman, unpublished data). HEL-DR cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The MO7e cell line, a factor-dependent cell line established from a patient with acute megakaryocytic leukemia (Avanzi et al., 1988), was maintained in Dulbecco's Modified Eagle's Medium (DMEM)/10% FBS with IL-3 as growth stimulus (1/50 dilution of transfected CHO cell conditioned medium). The factor-dependent human erythroleukemia cell line TF-1 (Kitamura et al., 1989), obtained from Dr. Douglas Williams (Immunex Corporation, Seattle, WA), was maintained in RPMI-1640/10% FBS with granulocyte macrophage-colony stimulating factor (GM-CSF) as growth factor (1/50 dilution of transfected CHO cell conditioned medium). Trans-

fected CHO cells stably expressing human IL-3 or GM-CSF were provided by Dr. Angel Lopez, Hanson Centre. Recombinant human (rh) SLF was obtained as purified material produced in yeast from Immunex (MGF) or in *E. coli* from Amgen Corporation, Thousand Oaks, CA (SCF) as indicated.

Immunofluorescence and flow cytometry

Procedures for detecting MAb binding to cells were as previously described (Bühring et al., 1991b; Ashman et al., 1991). Briefly, cells were incubated with anti-P145$^{c\text{-}kit}$ or control MAbs at 4°C for 30 minutes, washed twice, then incubated for a further 30 minutes with fluorochrome-conjugated (F(ab)'$_2$ fragments of goat antibodies specific for mouse immunoglobulin isotypes, as indicated. In order to block binding mediated by Fc receptors, cells were preincubated for 10 minutes with human AB serum (Behring, Marburg, Germany), or 10% heat-inactivated normal rabbit serum was included in the incubations. Except where otherwise indicated, assays were carried out in the presence of sodium azide (0.1%) to prevent capping and receptor internalization. To measure SLF binding, cells were incubated with a saturating level (125 μg/ml) of biotinylated SLF (MGF-b) at 4°C for 30 minutes, then washed and incubated with Streptavidin-phycoerythrin (SA-PE, Dianova, Hamburg, Germany) as above. Flow cytometric analysis was performed using a FACS IV cell sorter, a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), or a Profile II flow cytometer (Coulter, Hialeah, FL) as previously described (Bühring et al., 1991b; Ashman et al., 1991).

Receptor phosphorylation

MO7e cells (1.7×10^8) were washed once in phosphate-free RPMI-1640 (Irvine Scientific, Santa Ana, CA) supplemented with 0.5% bovine serum albumin (Reheis, Phoenix, AZ), then resuspended in 20 ml of phosphate-free RPMI containing 0.5% bovine serum albumin and 10 mCi ^{32}P-orthophosphate (Amersham, Arlington Heights, IL). After 3.5 hours of incubation in a shaking water bath maintained at 37°C, aliquots of the cells (2.4×10^7) were treated with no addition, SLF (rh-SCF at a final concentration of 50 ng/ml), or purified MAb (final concentration 5 μg/ml) for 15 minutes at room temperature. The samples were centrifuged for 1 minute in a Beckman Microfuge 11 (Beckman, Palo Alto, CA) operated in a 4°C cold room, and the cell pellets were lysed by rocking for 20 minutes at 4°C in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin, 200 μg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, 100 mM NaF). The method was based on that of Lev et al. (1992a). The Triton X-100, leupeptin, and aprotinin were obtained from Boehringer Mannheim (Indianapolis, IN) the remaining chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The cell lysates were centrifuged for 5 minutes at 10,000g to remove insoluble debris, and cellular proteins were precipitated overnight at 4°C with protein A-agarose beads (Pharmacia LKB, Piscataway, NJ) that had been coated with the 4G10 antiphosphotyrosine MAb (Siegel, 1992). The immunoprecipitates were washed once with HNTG buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), then adsorbed proteins were eluted by incubating the beads in HNTG containing 50 mM phenyl phosphate (Sigma) for 1 hour at 4°C (Lev et al., 1992a). The proteins were reprecipitated with the SR-1 MAb (1 μg/ml final concentration) and protein A-agarose beads. The immunoprecipitates were washed once in HNTG buffer, resuspended in sodium dodecyl sulfate (SDS) polyacrylamide sample buffer containing 50 mM dithiothreitol (Laemmli, 1970), boiled for 3 minutes, and analyzed on a 7.5% SDS polyacrylamide gel in parallel to prestained molecular weight markers (Bio-Rad Laboratories, Richmond, CA). Radiolabeled proteins were detected by autoradiography for 5 days at −70°C. Radioactivity in the dried gel was quantitated using a Molecular Dynamics PhosphorImager Model 400S (Sunnyvale, CA).

MO7e proliferation assay

MO7e cells cultured in the presence of IL-3 were washed twice in DMEM + 2% FBS to remove factor, then plated at 10^4 cells per well in 96 well flat bottomed microtiter plates (Falcon no. 3072, Becton Dickinson, New Jersey). Cells were preincubated with purified sterile, azide-free MAb at 2 μg/ml or 5 μg/ml for 1 hour at 37°C prior to the addition of SLF (0–50 ng/ml). Cultures were incubated at 37°C in a humidified 5% CO_2 in air atmosphere for 3 days and pulsed with 1 μCi/well methyl-^{3}H-thymidine (25 Ci/mmol, Amersham) for the last 16 hours of the culture period. Cells were harvested with a Titertek harvester and the filter discs placed in Hisafe scintillation fluid (Linbrook International) and counted in a Beckman LS7500 scintillation counter.

RESULTS

Competitive binding of YB5.B8, 17F11, and SR-1 to HEL-DR cells

Aliquots of HEL-DR cells were preincubated with each of the anti-P145$^{c\text{-}kit}$ MAbs under conditions preventing receptor internalization (4°C, in the presence of sodium azide) prior to the addition of the test antibody. Since the three antibodies are of different isotypes, binding of the test antibody was then assessed using an appropriate isotype-specific second-stage reagent. As shown in Figure 1, all three MAbs bound similarly to HEL-DR cells. Binding of SR-1 was not influenced by preincubation with YB5.B8 or 17F11. Conversely, SR-1 did not affect the binding of the other antibodies. Thus, as was previously reported for YB5.B8 (Broudy et al., 1992a), SR-1 identifies an epitope distinct from those recognized by the other antibodies. While preincubation with 17F11 had minimal effect on the subsequent binding of YB5.B8 to HEL-DR cells (average 6% inhibition based on mean fluorescence intensity), preincubation with YB5.B8 substantially blocked subsequent binding of 17F11 (average 60% inhibition). The results suggest that these two antibodies bind to distinct epitopes that may interact by way of conformational changes induced by antibody binding, although effects of antibody affinity have not been ruled out. Preincubation with control antibodies of the appropriate isotype and directed against HLA class I antigens, or irrelevant (anti-*Salmonella*) antigens, did not influence binding of anti-P145$^{c\text{-}kit}$ antibodies in any case. Similar results

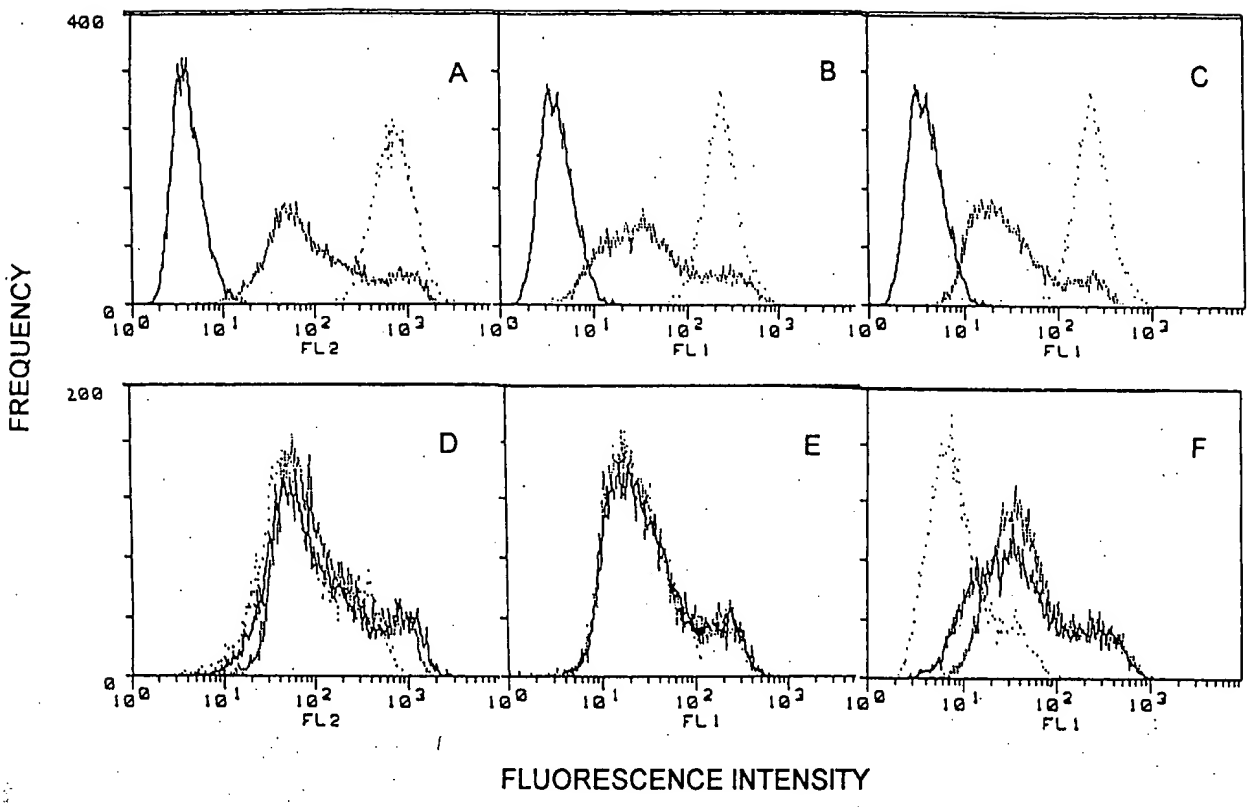

Fig. 1. Competitive binding of YB5.B8, 17F11, and SR-1 to HEL-DR cells. Binding of MAbs was measured by indirect immunofluorescence and analysis on a FACScan flow cytometer. A–C: Binding of MAbs YB5.B8, SR-1, and 17F11, respectively, to HEL-DR cells. —— isotype-matched negative control; – – – anti-kit MAb; · · · · isotype-matched anti-HLA class I (positive control). D–F: Effect of preincubating the cells with the other anti-*kit* MAbs. D: Binding of YB5.B8 alone (——) or after preincubation with SR-1 (– – –) or 17F11 (· · · ·); E: binding of SR-1 alone (——) or after incubation with YB5.B8 (– – –) or 17F11 (· · · ·); F: binding of 17F11 alone (——) or after preincubation with SR-1 (– – –) or YB5.B8 (· · · ·). YB5.B8 and control IgG1 MAbs were detected with PE-labeled goat anti-mouse IgG1; SR-1 and control IgG2a MAbs with FITC-labeled goat anti-mouse IgG2a, and 17F11 and control IgM MAbs with FITC-labeled goat anti-mouse IgM. All second-stage reagents used in this experiment were obtained from Caltag (San Francisco, CA).

were obtained using primary AML blasts and MO7e cells (data not shown).

Binding of YB5.B8, 17F11, and SR-1 to normal human bone marrow cells

Because of the slight differences in the documented specificity of the three antibodies, we compared their binding to bone marrow mononuclear cells from three normal human donors using two-color immunofluorescence and flow cytometry. Cell populations were incubated with YB5.B8 and SR-1 or 17F11 and SR-1 followed by isotype-specific second-stage reagents (as above). YB5.B8 and 17F11 could not be compared directly because they partially cross-block each other's binding. In each case the three antibodies bound to the same cell populations. A representative experiment is shown in Figure 2.

Influence of MAbs on SLF binding and vice versa

HEL-DR and MO7e cells, preincubated with anti-P145$^{c\text{-}kit}$ MAbs or isotype-matched control MAbs under conditions preventing receptor endocytosis, were assayed for binding of biotin-labeled SLF by immunofluorescence and flow cytometry. MAb SR-1 at 2 μg/ml almost completely abolished binding of SLF to both cell lines (Table 1). Binding of SLF to HEL-DR cells was unaffected by pretreatment with 17F11, and actually enhanced on MO7e cells. MAb YB5.B8 had a slight, but reproducible inhibitory effect on SLF binding on all three cell targets (Table 1). In the reciprocal experiment, preincubation of the cells with SLF (125 ng/ml) substantially inhibited SR-1 binding and slightly inhibited YB5.B8 binding, but enhanced 17F11 binding to primary AML cells. However, the results obtained with other cell types varied considerably. Surprisingly, preincubation with SLF influenced binding of YB5.B8 and SR-1 to HEL-DR cells and MO7e cells to a similar extent. SLF at up to 200 ng/ml was relatively ineffective at blocking SR-1 binding to MO7e cells (Table 2).

Effect of MAbs on receptor endocytosis

MO7e cells were preincubated with azide-free anti-P145$^{c\text{-}kit}$ or control MAb at 4°C then SLF was added, the cells warmed to 37°C, and cell surface P145$^{c\text{-}kit}$ was monitored after the indicated times by indirect immunofluorescence. Figure 3 shows the results of a representative experiment where SLF (50 ng/ml) brought

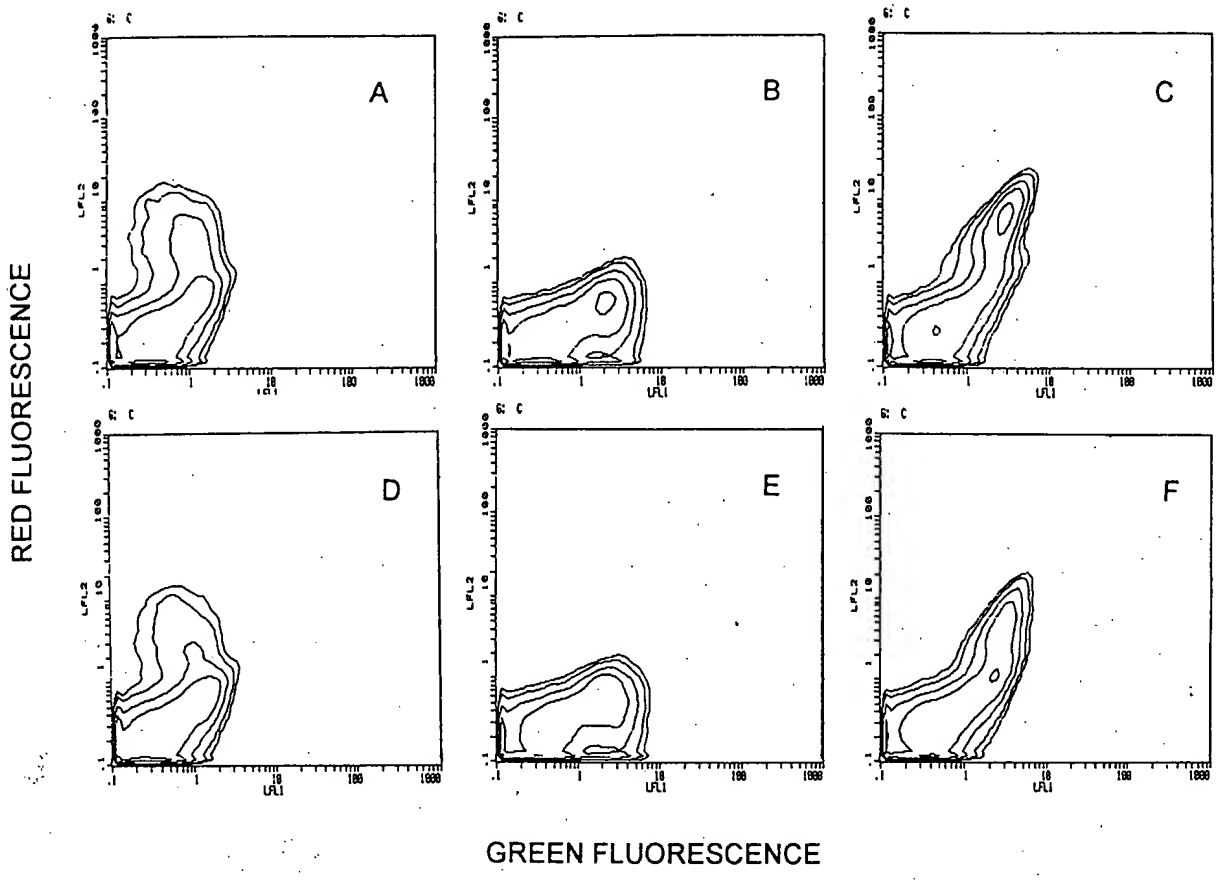

Fig. 2. Binding of YB5.B8, SR-1, and 17F11 to normal bone marrow cells. Subsets of low density bone marrow cells were assayed by two-color indirect immunofluorescence and analyzed on a Profile II flow cytometer. A: YB5.B8 + IgG2a negative control; B: IgG1 negative control + SR-1; C: YB5.B8 + SR-1; D: 17F11 + IgG2a negative control; E: IgM negative control + SR-1; F: 17F11 + SR-1. All primary antibodies were used at 5 μg/ml final. YB5.B8 was detected with PE-labeled anti-IgG1; SR-1 with FITC-labeled anti-IgG2a (c.f. Fig. 1 legend); and 17F11 with PE-labeled goat anti-mouse IgM (Southern Biotechnology).

TABLE 1. Effect of MAbs on Binding of SLF[1]

	Relative mean fluorescence intensity		
MAb	AML cells	HEL-DR cells	MO7e cells
None	100	100	100
SR-1	1	1	1
YB5.B8	57	89	81
17F11	107	104	130

[1]Cells were incubated with MAb 2 μg/ml for 30 minutes on ice in the presence of 0.01% NaN_3 prior to the addition of biotinylated SLF (rhMGF). After a further 30 minutes on ice, cells were stained with Streptavidin-PE and analyzed by flow cytometry using linear gain.

TABLE 2. Effect of SLF on MAb Binding[1]

	Relative mean fluorescence intensity		
MAb	AML cells	HEL-DR cells	MO7e cells
No SLF	100	100	100
SR-1	26	42	85
YB5.B8	80	42	89
17F11	156	111	101

[1]Cells were incubated in medium or with SLF (rhSCF) 125 ng/ml (AML cells) or 200 ng/ml (HEL-DR and MO7e cells) for 10 minutes on ice in the presence of 0.01% NaN_3 prior to incubation with the indicated MAb (2 μg/ml) for a further 30 minutes on ice. Subsequently the cells were stained with fluorescent-labeled antibodies to mouse Ig and analyzed by flow cytometry using linear gain. For each antibody the mean fluorescence intensity observed with cells preincubated with SLF is expressed relative to the control (no SLF), assigned the arbitrary value of 100.

about essentially complete receptor down-regulation by 30 minutes of incubation. MAb 17F11 also caused strong down-regulation of cell surface $P145^{c\text{-}kit}$. In contrast, incubation with SR-1 caused only modest loss of the receptor but blocked SLF-induced receptor modulation, while YB5.B8 had a negligible effect alone or on the response to SLF. Similar results were obtained with HEL-DR cells (not shown).

Receptor phosphorylation induced by binding of MAbs to MO7e cells

MO7e cells, metabolically labeled with ^{32}P-orthophosphate, were incubated with SLF, anti-$P145^{c\text{-}kit}$, or control MAb for 15 minutes then solubilized. After sequential immunoprecipitation with 4G10 antiphosphotyrosine MAb followed by SR-1 MAb and SDS-polyacrylamide gel electrophoresis (PAGE), the extent of phosphorylation of $P145^{c\text{-}kit}$ was determined by autoradiography (Fig. 4) and PhosphorImager analysis of the major band at approximately 150 KDa. Basal phosphorylation of $P145^{c\text{-}kit}$ was detectable in control MO7e cells in the absence of SLF or MAb. Incubation with SLF increased receptor phosphorylation 9.2-fold, in

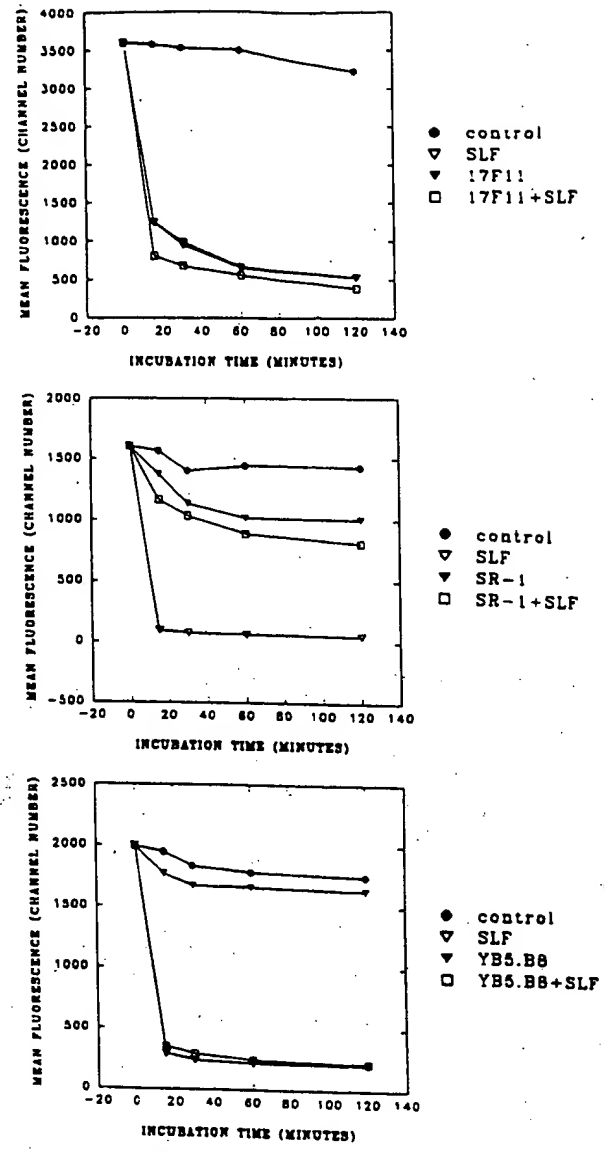

Fig. 3. Effect of MAbs on receptor internalization. MO7e cells were preincubated with SLF (rh-SCF) and/or MAbs (2 μg/ml) for 30 minutes at 4°C, then rapidly warmed to 37°C. At the indicated times, aliquots were removed and residual P145$^{c\text{-}kit}$ was determined by indirect immunofluorescence. A: 17F11; B: SR-1; C: YB5.B8. ●, control cells; ▽, SLF alone; ▼, MAb alone; [▫] MAb + SLF.

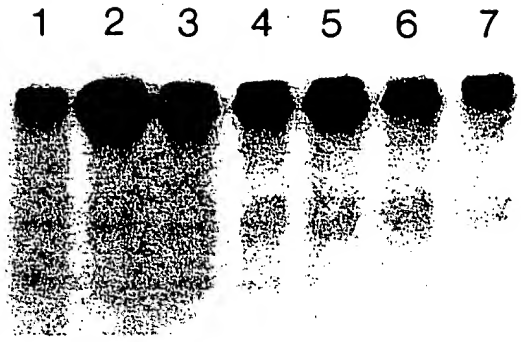

Fig. 4. Effect of MAbs on receptor phosphorylation in intact cells. MO7e cells were metabolically labeled with ^{32}P-orthophosphate, then incubated for 15 minutes with no addition (lane 1), SLF (rh-SCF, 50 ng/ml) (lane 2), SR-1 (lane 3), YB5.B8 (lane 4), 17F11 (lane 5), 3D6 (lane 6), or a negative control MAb SAL-2 (lane 7). Each of the MAbs was used at a concentration of 5 μg/ml. The cells were then lysed, immunoprecipitated with antiphosphotyrosine MAb 4G10, and then reprecipitated with SR-1. After SDS-PAGE, the labeled proteins were detected by autoradiography.

comparison to control MO7e cells. With the exception of the negative control antibody SAL-2, all of the antibodies tested brought about some increase in P145$^{c\text{-}kit}$ phosphorylation. This was greatest for 17F11 (3.4-fold), followed by SR-1 (3.1-fold), YB5.B8 (2.0-fold), and least (1.8-fold) for 3D6, another anti-P145$^{c\text{-}kit}$ MAb (Bühring et al., 1993).

Effect of MAbs on proliferation of MO7e cells

MO7e cells, previously maintained in rhIL-3, were cultured with various levels of SLF and anti-P145$^{c\text{-}kit}$ MAb or isotype-matched negative control MAb. Proliferation was assessed by ^{3}H-thymidine incorporation after 3 days of culture. Maximum proliferation was observed at a SLF concentration of 25 ng/ml (rhMGF; Immunex). As expected, SR-1 almost completely blocked SLF-driven MO7e proliferation. Results of a representative experiment are shown in Figure 5. YB5.B8 had no significant inhibitory effect at any concentration of SLF tested, and 17F11 caused partial inhibition (average 50%) at all levels of SLF. Similar results were obtained with TF-1 cells (data not shown). In the absence of added SLF, slight but reproducible enhancement of thymidine incorporation was observed with YB5.B8 and 17F11, but not with SR-1. This was on average 5.2 ± 2.6% of the value obtained with 25 ng/ml SLF for 17F11, and 4.9 ± 2.4% for YB5.B8, after correcting incorporation for the isotype-matched control (n = 5 in each case). These two MAbs also enhanced thymidine incorporation by TF-1 cells (6.3 ± 2.2% and 3.3 ± 1.2% of the value obtained with 25 ng/ml SLF for 17F11 and YB5.B8 respectively; n = 4).

DISCUSSION

As outlined above, all three MAbs studied here have previously been clearly demonstrated to bind to human P145$^{c\text{-}kit}$, but there appeared to be some differences in their specificity. Direct comparison revealed that these MAbs bound to indistinguishable cell populations in normal bone marrow and, while variations in specificity due to alternate glycosylation of the P145$^{c\text{-}kit}$ on different cell types cannot be excluded, it seems likely that the previously reported differences in the binding of these antibodies to leukemic cell populations reflect the varying sensitivity of the techniques employed. However, the antibodies recognized distinct epitopes on the extracellular domain of P145$^{c\text{-}kit}$. In cross-blocking experiments, SR-1 was shown to bind to an epitope independent of those recognized by YB5.B8 and 17F11. While some cross-blocking between YB5.B8 and 17F11 was observed, this was incomplete and nonreciprocal; YB5.B8 was much more effective at blocking 17F11 binding than vice versa in experiments where cells were incubated sequentially with the two antibodies. Although the results could be explained by substantial

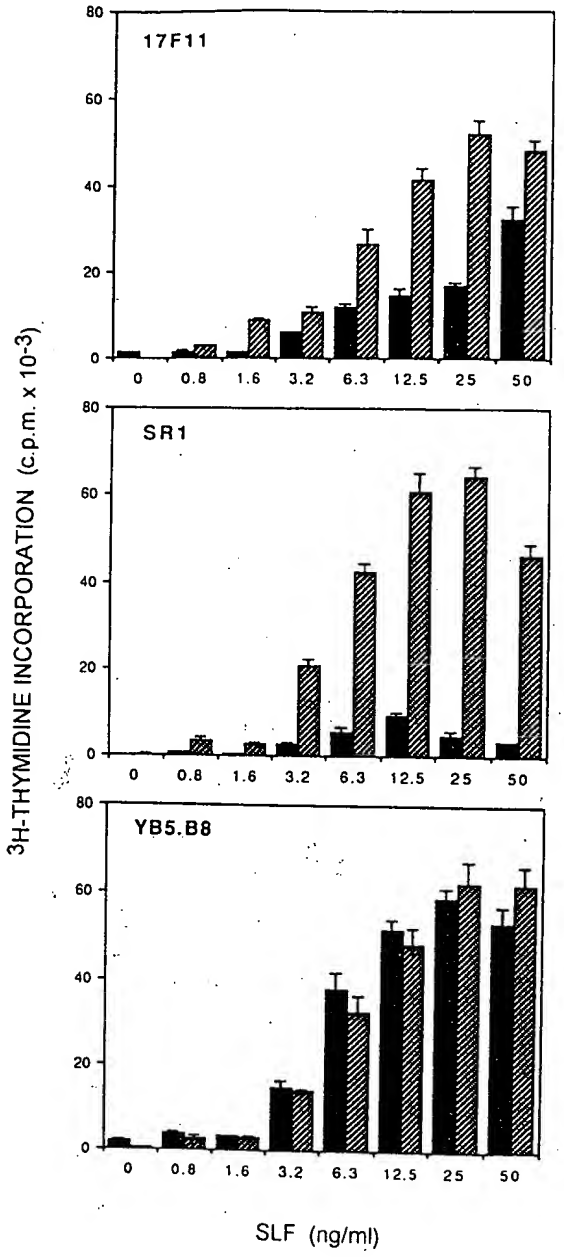

Fig. 5. Effect of MAbs on the proliferation of MO7e cells. Cells were cultured for 3 days with the indicated level of SLF (rh-MGF) in the presence of anti-P145$^{c\text{-}kit}$ MAbs (solid bars) or isotype-matched control MAb (hatched bars) (all at 5 μg/ml final concentration, except SR-1 and the negative control IgG2a which were used at a 1/5,000 final dilution of ascites). Proliferation was measured by incorporation of ^{3}H-thymidine over the last 16 hours of culture. All cultures were performed in quadruplicate and the histograms show means and S.E.

differences in affinity, it seems likely that the two antibodies bind to separate epitopes, and that YB5.B8 inhibits 17F11 binding by inducing a conformational change in the receptor.

P145$^{c\text{-}kit}$ is a member of the type III receptor tyrosine kinase family and displays strong homology with the CSF-1 receptor (Ullrich and Schlessinger, 1990). Signal transduction by receptor tyrosine kinases involves the ligand-induced formation of receptor homodimers, stimulation of the intrinsic kinase activity, auto and/or transphosphorylation, association with and phosphorylation of downstream signaling molecules culminating in mitogenesis, and finally endocytosis and degradation of the receptor-ligand complex. In the case of P145$^{c\text{-}kit}$, binding of the ligand SCF, which exists in solution as a dimer (Arakawa et al., 1991), is believed to promote receptor dimerization either directly (Blume-Jensen et al., 1991) or by means of a conformational change (Lev et al., 1992b), resulting in activation of the kinase function of the cytoplasmic domain of the receptor. Thus MAbs to the extracellular part of P145$^{c\text{-}kit}$ may modify its function by blocking or mimicking ligand binding, or influencing receptor dimerization. The effects could be either by direct competition for ligand or homodimer binding sites, or mediated by conformational changes. In this study, the effects of MAbs on receptor function were assessed by examination of their effects on ligand binding, receptor phosphorylation in intact cells, receptor down-regulation, and mitogenesis in factor-dependent MO7e cells.

SR-1 was the only antibody to significantly influence ligand binding, and appeared to directly compete with SLF for binding to the receptor since reciprocal, essentially complete, inhibition was observed at least on some cell types. Although it induced significant receptor phosphorylation in intact cells, and weakly promoted receptor endocytosis, it was devoid of agonist activity in the MO7e proliferation assay. These results imply that receptor phosphorylation and internalization can be to some extent dissociated from mitogenesis. Whether the pattern of tyrosine phosphorylation induced by SR-1 differs qualitatively as well as quantitatively from that brought about by SLF remains to be determined.

The biological effects of 17F11 were more complex. This antibody failed to compete for ligand binding, but appeared to have slight, but significant agonist activity. It brought about the highest level of receptor phosphorylation (37% of that seen with SLF), synergized with SLF in promoting receptor internalization, and induced some proliferation of MO7e cells in the absence and at very low levels of ligand, but inhibited proliferation at near-saturating levels of SLF. In other studies on primary AML cells (Bühring et al., 1993), 17F11 was shown to synergize with SLF. Since the antibody does not appear to bind to the ligand binding site, it may influence function through effects on receptor oligomerization.

MAb YB5.B8 was previously shown to block the binding of murine SLF to human erythroleukaemia (HEL) cells (Lerner et al., 1991), but had little effect on the binding of rhSLF (rhMGF) to HEL-DR or MO7e in the experiments reported here. Since YB5.B8 did not cross-block with SR-1, which appears to directly compete for the ligand binding site of P145$^{c\text{-}kit}$, the results imply that any effect of YB5.B8 on ligand binding is mediated by a conformational change, or that the recognition site for murine SLF on P145$^{c\text{-}kit}$ is nonidentical with the binding site for hSLF. Consistent with the latter interpretation, Lev et al. (1993) recently described experiments using chimeric human-mouse P145$^{c\text{-}kit}$ showing that the hSLF binding site is located on the second

Ig-like domain of the receptor, while the rodent SLF binding site was predominantly dependent on the third Ig-like domain. Nevertheless, the nonreciprocal cross-blocking between YB5.B8 and 17F11, described above, provides evidence that YB5.B8 binding does indeed induce a conformational change in the receptor, although the possibility that it is due to differences in affinity has not been excluded. YB5.B8 has been shown to have some inhibitory activity in a range of culture systems of normal hemopoietic cells in several laboratories (Cambareri et al., 1988; Ashman et al., 1990; Cicuttini et al., 1992; Matos et al., 1993). In addition, blocking of YB5.B8 binding by preincubation with SLF varied considerably for different target cells (Table 2). These results imply that there are conformational interactions between the SLF and YB5.B8 binding sites on the receptor, and that these interactions vary in different cell types. At present we have no explanations for the discrepancies between the results obtained with different cell types, but it should be noted that the extent of binding of YB5.B8 is indistinguishable from that of the other MAbs to $P145^{c\text{-}kit}$ on all cell types examined. It is possible that YB5.B8 has differential effects depending on receptor glycosylation, the balance of $P145^{c\text{-}kit}$ isoforms (Reith et al., 1991; Hayashi et al., 1991; Crosier et al., 1993) expressed by the target cell, or whether the ligand is functioning in soluble or membrane-bound form (Anderson et al., 1990; Flanagan et al., 1991; Huang et al., 1992).

Thus, the three anti-MAbs studied in these experiments have distinctive properties. As well as having different isotypes, the antibodies bound to separate epitopes on the extracellular domain of $P145^{c\text{-}kit}$ and had distinct biological effects. Although 17F11 did not compete with SLF for binding to the receptor, it had the characteristics of a weak agonist in its interactions with cells expressing $P145^{c\text{-}kit}$. Of the three antibodies, it was the only one to promote effective receptor down-modulation, presumably by internalization. It may, therefore be the most suitable of the antibodies tested here for immunotoxin delivery. In contrast, SR-1 was a potent antagonist of SLF binding, and is clearly the best of the three antibodies for use as an inhibitor of SLF action. In this respect it may be similar to another recently described MAb 3D6 (Bühring et al., 1993). Notwithstanding its inhibitory action on SLF binding, SR-1 has been successfully used to isolate primitive hemopoietic cells including long-term bone marrow culture initiating cells (Briddell et al., 1992) and pre-CFU (Papayannopoulou et al., 1991). MAb YB5.B8 was the most neutral in terms of its effects on SLF-mediated responses. It had little effect on ligand binding, receptor status (phosphorylation, down-regulation), or on SLF-induced proliferation of MO7e cells. It is therefore an ideal antibody for the isolation of $P145^{c\text{-}kit}$ expressing cells such as mast cells and early stem cells. We have demonstrated excellent yields of CFU over several weeks in cultures of pre-CFU isolated with YB5.B8 by FACS (Simmons et al., 1994). In addition, YB5.B8 has been used, with immunomagnetic beads, in the purification of tissue mast cells in high yield and without functional alteration (Bradding et al., 1992; Okayama et al., 1994).

ACKNOWLEDGMENTS

We thank M. Burkhardt for excellent technical assistance. We are grateful to Drs. D. Williams, P. DeVries, and S. Gillis, Immunex Corporation, Seattle, WA, for providing rhMGF and its biotinylated derivative, and to Dr. K. Zsebo, Amgen Corporation, Thousand Oaks, CA for rhSCF. This work was supported by grants from the Anti-Cancer Foundation of the Universities of South Australia (to L.K.A.), the Deutsche Forschungsgemeinschaft (SFB 120, project C5 to H.-J.B.), and the National Institutes of Health (DK 44194 to V.C.B.). L.K.A. is a Senior Research Fellow of the National Health and Medical Research Council of Australia. V.C.B. is also supported by a Faculty Research Award from the American Cancer Society.

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